Phosphorylation of the translational regulator, PHAS-I, by protein kinase CK2

Patrick Fadden, Timothy A.J. Haystead, John C. Lawrence Jr.*

Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Health Sciences Center, Box 448, Charlottesville, VA 22908, USA

Received 9 June 1998; revised version received 7 August 1998

Abstract The primary site in PHAS-I for phosphorylation by protein kinase CK2 in vitro was identified as Ser^{111}. A relatively small amount of phosphorylation of Ser^{99} was also detected, and mutating Ser^{99} to Ala in PHAS-I slightly decreased phosphorylation by CK2 in vitro. In contrast, mutating Ser^{111} to Ala almost abolished phosphorylation, confirming Ser^{111} as the preferred site for CK2. Phosphorylation of Ser^{111} did not decrease binding of PHAS-I to eIF4E, and results of peptide mapping experiments with PHAS-I immunoprecipitated from ^{32}P-labeled adipocytes indicated that Ser^{111} was not phosphorylated in cells. These results support the conclusion that CK2 is not involved in the control of PHAS-I.

© 1998 Federation of European Biochemical Societies.

Key words: Casein kinase II; 4E-BP1; PHAS-I; Insulin

1. Introduction

PHAS-I (also known as 4E-BP1) regulates mRNA translation initiation by controlling the availability of eIF4E [1–3]. This initiation factor is responsible for recognition of the mRNA cap (m^7GpppN, where N is any nucleotide), which is found at the 5′-end of almost all eukaryotic mRNAs. Non-phosphorylated PHAS-I binds tightly to eIF4E and prevents eIF4E from binding to eIF4G, a large scaffolding protein that also binds eIF4A and eIF3. When phosphorylated in the appropriate site(s), PHAS-I dissociates from eIF4E. This allows eIF4E to bind eIF4G to form a complex needed for efficient binding and/or scanning by the 40S ribosomal subunit. Insulin and growth factors increase mRNA translation by promoting the phosphorylation of PHAS-I [1–3]. In addition, the phosphorylation of PHAS-I may contribute to the mitogenic actions of growth factors, as increasing eIF4E has been shown to have a potent mitogenic effect, at least in some cell types [4,5]. For these reasons it is important to identify the protein kinases that mediate the phosphorylation of PHAS-I in cells.

Rat adipocytes express very high levels of PHAS-I and are exquisitely responsive to insulin [6]. Five phosphorylation sites, all having a (S/T)P motif, have been identified in PHAS-I from adipocytes [7]. The finding that the effect of insulin on increasing phosphorylation of PHAS-I was attenuated by rapamycin implicated mTOR in the control of the protein [8–10]. In recent studies all five (S/T)P sites were found to be phosphorylated by mTOR in vitro [11,12]. While it is clear that mTOR has an important role in controlling PHAS-I, there is reason to suspect that other kinases contribute, as rapamycin does not abolish the effect of insulin on increasing the phosphorylation of PHAS-I [10,13].

Protein kinase CK2, also known as casein kinase II, was the first protein kinase found to phosphorylate PHAS-I in vitro [14]. CK2 has the interesting property of phosphorylating the eIF4E-bound form of PHAS-I more rapidly than it phosphorylates free PHAS-I [13,15]. Insulin has been reported to increase the activity of CK2 in rat adipocytes [14], and it has been suggested that phosphorylation in the CK2 site primes PHAS-I in the eIF4E complex for phosphorylation by other protein kinases [3]. CK2 prefers an acidic residue immediately COOH terminal (+1 to +3) to the phosphorylated Ser/Thr residue [16,17]. PHAS-I contains four sites that meet this minimal consensus requirement [18]. The objectives of the present study were to identify sites in PHAS-I phosphorylated by CK2 in vitro and to determine whether the sites were phosphorylated in response to insulin in rat adipocytes.

2. Materials and methods

2.1. Preparation of recombinant PHAS-I proteins

Histidine-tagged PHAS-I ([H^6]PHAS-I) and mutant proteins were purified as described previously [19]. cDNAs encoding mutant PHAS proteins were generated using the polymerase chain reaction with mutagenic oligonucleotide primers and rat PHAS-I cDNA as template [18]. The primers were designed to yield products having *Bam*HI sites at both the 5′ and 3′ ends. Ser^{99}, Ser^{111}, and Ser^{99} plus Ser^{111} were mutated by converting the respective Ser codons to Ala codons in the 3′ primers. After amplification (20 cycles), the products were purified, cut with *Bam*HI, and inserted into the *Bam*HI site of pET-14b (Novagen). cDNA clones used to express proteins were sequenced to be sure that no unwanted mutations were present.

2.2. Purification of protein kinase CK2

CK2 was purified by a method similar to that described by Hathaway and Traugh [20]. Briefly, rabbit skeletal muscle (500 g, New Zealand White) was homogenized in Buffer A (1 mM EDTA, 1 mM DTT, 50 mM MOPS, pH 7.5) containing 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 μg/ml aprotinin. The homogenate was centrifuged at $11\,000 \times g$ for 30 min, and the supernatant was loaded onto a Fast Flow Q Sepharose column (200 ml) that had been equilibrated in Buffer A. Proteins were eluted with a 3-l linear gradient of NaCl (from 0 to 1 M) in Buffer A and fractions (15 ml) were collected. CK2 activity was measured (described later) and peak fractions were pooled, dialyzed against Buffer A plus 250 mM NaCl, and applied to a phosphocellulose column (40 ml) that had been equilibrated in the dialysis buffer. The column was washed with 10 volumes of Buffer A plus 450 mM NaCl. CK2 was eluted with Buffer A plus 800 mM NaCl. Peak fractions containing the kinase were pooled and dialyzed against Buffer B (1 mM EDTA, 1 mM DTT, 15 mM potassium phosphate, pH 6.8). The sample was applied to a 1-ml hydroxylapatite (Bio-Rad) column that had been equilibrated in Buffer B. The column was washed with 10 volumes of Buffer B before CK2 was eluted with a 150-ml linear gradient (from 15 mM to 300 mM) of sodium phosphate. Peak fractions were pooled, dialyzed against Buffer B plus 1 M NaCl, and stored at 4°C.

*Corresponding author. Fax: (1) (804) 982-3575.
E-mail: jcl3p@virginia.edu

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(98)01047-3

2.3. Measurements of CK2 activity

Samples (10 μl) were added to reaction mixtures (20 μl) that contained 150 mM NaCl, 7.5 mM $MgCl_2$, 300 mM [γ-^{32}P]ATP (~1000 cpm/pmol), 100 μM RRDDDSDDD, and 10 mM HEPES, pH 7.4. After incubating at 23°C for 20 min, the reactions were stopped by adding 20 μl of 1% phosphoric acid. The samples were then spotted onto squares (1 cm) of phosphocellulose paper (Whatman P81), which were washed with phosphoric acid as described previously [21].

2.4. Peptide mapping

Samples of ^{32}P-labeled PHAS-I were digested with lysyl endopeptidase (Wako) and peptides were resolved by reverse phase HPLC as described previously [7]. For cleavage with CNBr, samples of ^{32}P-labeled PHAS-I were dried under vacuum (Speedvac, Savant), then dissolved in 100 μl 70% formic acid. After adding 1 mg CNBr to each, the samples were incubated for 15 h at 21°C. The samples were then dried under vacuum, dissolved in 200 μl H_2O, taken to dryness again, and dissolved in either 0.1% trifluoroacetic acid for HPLC analyses or in SDS-sample buffer for SDS-PAGE [22].

2.5. Electrophoretic analyses

Phosphoamino acid analysis was conducted using the method of Geer et al. [23]. Far-Western analysis to assess binding of PHAS-I to eIF4E was performed as described previously [24]. Briefly, PHAS-I proteins were subjected to SDS-PAGE [22], then transferred to Immobilon (Millipore) and incubated with a ^{32}P-labeled FLAG-eIF4E fusion protein. After washing the membranes, the amount of ^{32}P-labeled FLAG-eIF4E bound was determined by autoradiography.

2.6. Amino acid sequencing

The ^{32}P-labeled peptides generated by phosphorylating recombinant PHAS-I with [γ-^{32}P]ATP and CK2 were identified by using a vapor phase amino acid sequencer (Applied Biosystems Procise 494). Phosphorylated residues within phosphopeptides were located by determining the cycles in which ^{32}P was released when samples were subjected to sequential Edman degradation under conditions that optimize recovery of ^{32}P [25].

2.7. Incubation of adipocytes and immunoprecipitation of PHAS-I

Rat adipocytes were isolated from male rats (Wistar, 120 g), incubated with $^{32}P_i$ and treated with insulin exactly as described previously [7]. [^{32}P]PHAS-I was immunoprecipitated from extracts by using an antibody directed against the COOH terminal region of PHAS-I [26].

3. Results

3.1. Phosphorylation of PHAS-I by CK2 does not inhibit eIF4E binding

Incubating recombinant PHAS-I for increasing times with purified CK2 and Mg-[γ-^{32}P]ATP resulted in a progressive incorporation of phosphate into the PHAS-I protein (Fig. 1). The stoichiometry approached 0.5 mol phosphate per mol PHAS-I after 2 h (Fig. 1). ^{32}P-Labeled FLAG-eIF4E binding to PHAS-I, assessed by far-Western blotting, was not decreased by the incubation with CK2, even after 2 h (Fig. 1).

3.2. Identification of phosphorylation sites

The results in Fig. 1 do not exclude CK2-mediated phosphorylation in the control of PHAS-I, as the sites phosphorylated by the kinase might modulate the effect of other phosphorylation sites on eIF4E binding. Therefore, experiments were performed to identify the sites in PHAS-I that were phosphorylated by CK2 and to determine whether these sites were phosphorylated in cells.

Thr^{84}, Ser^{99}, Ser^{100}, and Ser^{111} meet the minimal consensus requirements for phosphorylation by CK2 [16]. As a first step in determining which of these sites was phosphorylated, a sample of PHAS-I was subjected to acid hydrolysis and phosphoamino acids were separated by high voltage electrophoresis at pH 3.5. Phosphoserine was the only phosphoamino acid detected (Fig. 2A, inset). Therefore, Thr^{84} could be eliminated as a site of phosphorylation by CK2.

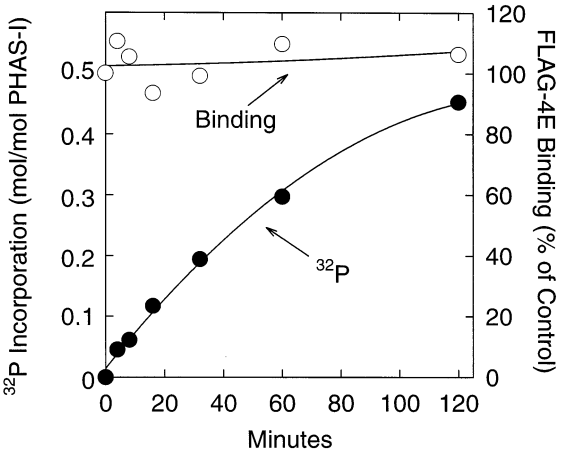

Fig. 1. Effect of increasing time of incubation with CK2 on PHAS-I phosphorylation and eIF4E binding. Samples of [H^6]PHAS-I (10 μg) were incubated at 23°C with 10 milliunits/ml CK2 (where 1 unit = 1 nmol phosphate incorporated into RRDDDSDDD in 1 min) in reaction mixtures (160 μl each) containing 150 mM NaCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), and either 300 μM ATP or 300 μM [γ-^{32}P]ATP (850 cpm/pmol). Samples (15 μl) were removed after increasing times, added to SDS-sample buffer (20 μl), and subjected to SDS-PAGE. ^{32}P incorporation (mol/mol [H^6]PHAS-I) was determined by scintillation counting of gel slices containing [H^6]PHAS-I. To assess eIF4E binding, proteins from samples incubated with unlabeled ATP were electrophoretically transferred to an Immobilon P membrane, which was then incubated with ^{32}P-labeled FLAG-eIF4E fusion protein (200 000 cpm/ml) as described previously [24]. The relative amounts of ^{32}P-labeled FLAG-eIF4E bound were determined by optical density scanning of an autoradiogram. The results are expressed as percentages of the ^{32}P-labeled FLAG-eIF4E bound to [H^6]PHAS-I that was not exposed to CK2.

To identify the phosphorylated Ser residues, PHAS-I was cleaved with CNBr. When samples were subjected to reverse phase HPLC to resolve peptides, a peak of ^{32}P eluting at approximately 40% acetonitrile was observed (Fig. 2A). Amino acid sequencing indicated that the phosphopeptide in this peak resulted from cleavage of PHAS-I at Met^{90} (Fig. 2B). Although the peptide was sequenced through only 8 cycles, the predicted phosphorylated fragment extends to Met^{115}. Thus, the fragment most likely contained all three Ser residues that were candidates for phosphorylation by CK2. In a separate sequencing run, the release of ^{32}P at each cycle of Edman degradation was determined. A surge of ^{32}P release was detected at cycle 9 (Fig. 2B). ^{32}P release in subsequent cycles then declined rapidly. These results indicate that Ser^{99} was a site of phosphorylation, although we cannot exclude the possibility that there was a relatively small amount of phosphate in Ser^{100}. However, the total amount of ^{32}P released through cycle 12 represented a very small fraction of the ^{32}P applied to the sequencer, suggesting that the phosphorylated CNBr peptide contained another phosphorylated residue. Through the process of elimination, this site would most likely be Ser^{111}.

Incubating PHAS-I with lysyl endopeptidase cleaves at Lys^{103} to generate a fragment containing Ser^{111}, which is located near the COOH terminus of the protein [18]. This fragment was the major phosphopeptide from CK2-phosphory-

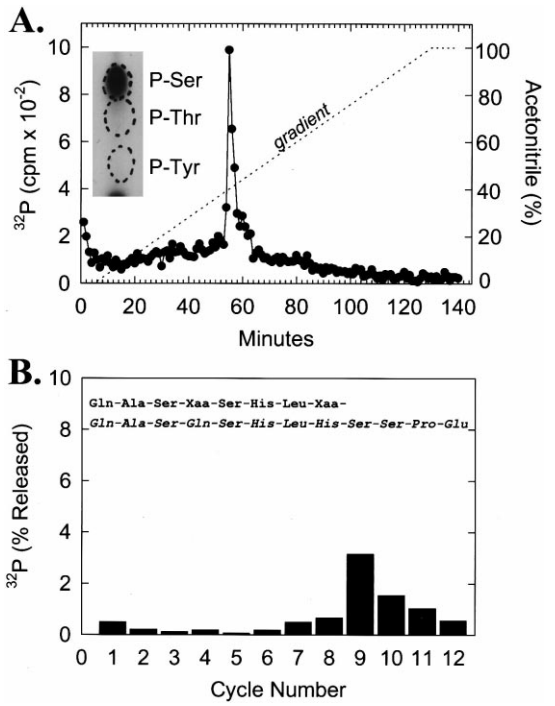

Fig. 2. Identification of Ser^{99} as a minor phosphorylation site CK2. A: [H^6]PHAS-I (25 μg) was phosphorylated by incubation with [γ-^{32}P]ATP and CK2 (1 unit/ml) at 23°C for 90 min. The protein was then cleaved with CNBr and the resulting fragments were resolved by reverse phase HPLC. Results represent the amount of ^{32}P recovered in each fraction. For phosphoamino acid analysis, [H^6]PHAS-I was phosphorylated by incubating with CK2 and [γ-^{32}P]ATP for 1 h at 23°C. The phosphorylated protein was purified by SDS-PAGE, then subjected to partial acid hydrolysis (5.7 N HCl for 1 h at 110°C). Phosphoamino acids were resolved by electrophoresis at pH 3.5. The positions of standards are indicated on the autoradiogram presented in the inset. B: The ^{32}P-labeled CNBr fragment was purified by reverse phase HPLC and subjected to 12 cycles of sequential Edman degradation. The amino acid sequence obtained is presented above the corresponding sequence (shown in italics) encoded by PHAS-I cDNA. In a separate sequencing run, the amount of ^{32}P released in each cycle was determined. Results are expressed as percentages of the total ^{32}P loaded.

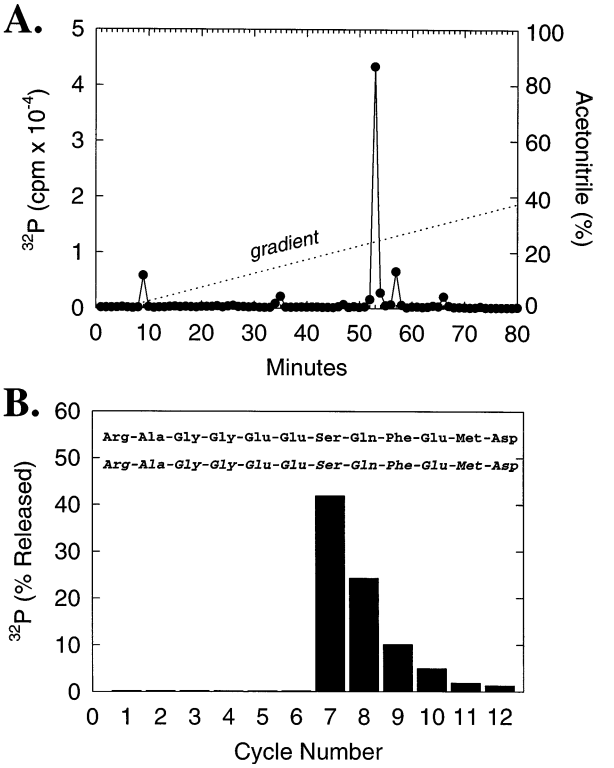

Fig. 3. Identification of Ser^{111} as the preferred site of phosphorylation by CK2. A: [H^6]PHAS-I was phosphorylated by incubation with [γ-^{32}P]ATP and CK2 (1 unit/ml) at 23°C for 90 min. The protein was then digested with lysyl endopeptidase and the resulting ^{32}P-labeled phosphopeptides were resolved by reverse phase HPLC. The amount of ^{32}P found in each fraction is presented. B: The major ^{32}P-labeled fragment obtained after lysyl endopeptidase digestion of PHAS-I was purified by reverse phase HPLC. The amino acid sequence of the peptide and amount of ^{32}P released in each cycle are presented as described in the legend to Fig. 2.

lated PHAS-I. It eluted from the reverse phase column in a sharp peak at 53 min (Fig. 3A), and its identity was established by amino acid sequencing (Fig. 3B). The surge in ^{32}P release occurred in cycle 7 (Fig. 3B), indicating that Ser^{111} was the phosphorylated residue.

To investigate further the phosphorylation of Ser^{99} and Ser^{111} by CK2, mutant PHAS-I proteins were prepared. As expected, CK2 introduced almost no ^{32}P into the PHAS-I protein in which both Ser^{99} and Ser^{111} had been mutated to Ala (Fig. 4). Changing Ser^{99} to Ala had a relatively small effect on the phosphorylation of PHAS-I, supporting the conclusion that Ser^{99} is not a preferred site of phosphorylation by CK2. In contrast, mutating Ser^{111} markedly decreased PHAS-I phosphorylation (Fig. 4), confirming the assignment of Ser^{111} as the major site of phosphorylation.

3.3. *Ser^{111} is not phosphorylated in rat adipocytes*

To determine whether Ser^{111} was phosphorylated in fat cells, samples of PHAS-I phosphorylated in vitro by CK2 and of PHAS-I immunoprecipitated from ^{32}P-labeled rat adipocytes were digested with lysyl endopeptidase and the resulting phosphopeptides were resolved by reverse phase HPLC (Fig. 5). The antibodies used immunoprecipitate both free and eIF4E-bound forms of PHAS-I [26], as well as PHAS-I phosphorylated in vitro by CK2 (Fadden, Haystead, and

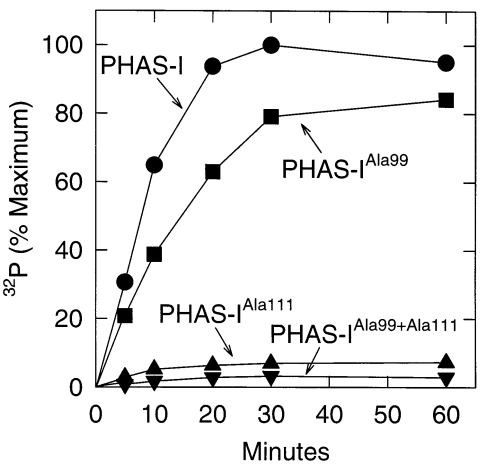

Fig. 4. Comparison of the rates of phosphorylation of PHAS-I and mutant PHAS-I proteins by CK2. PHAS proteins (10 μg each) were incubated with CK2 for increasing times as described in the legend to Fig. 1. The relative amounts of ^{32}P incorporated into the proteins were determined and are expressed as percentages of the maximum incorporation observed with [H^6]PHAS-I.

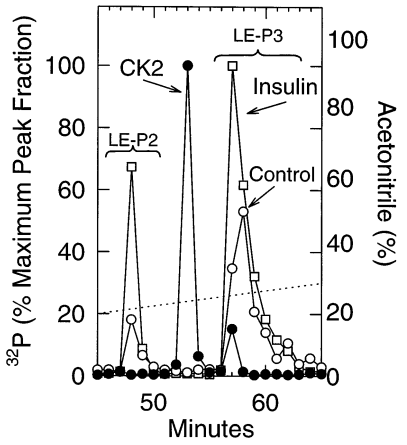

Fig. 5. Peptide mapping of PHAS-I phosphorylated in adipocytes and in vitro by CK2. Samples of PHAS-I from control and insulin-treated ^{32}P-labeled adipocytes, and [H^{6}]PHAS-I that had been phosphorylated with CK2, were digested with lysyl endopeptidase. Phosphopeptides were resolved by reverse phase HPLC, and the amount of ^{32}P in each fraction was determined. The results are expressed as percentages of the respective peak fractions from the in vitro and in vivo phosphorylated proteins.

Lawrence, unpublished observations). With the CK2-phosphorylated sample, the major peak containing Ser^{111} eluted between two peaks, previously designated LE-P2 and LE-P3 [7], from the adipocyte PHAS-I. LE-P2 contains a peptide phosphorylated in Ser^{64}; LE-P3 contains peptides phosphorylated in Ser^{36}, Ser^{45}, and Ser^{82}. A relatively small peak from the CK2 sample eluted in the position of LE-P2. This peak presumably contains Ser^{99}, which is found in the same lysyl endopeptidase peptide as Ser^{82} [7].

The absence of a peak of ^{32}P corresponding to the Ser^{111} peptide in PHAS-I from ^{32}P-labeled adipocytes implies that Ser^{111} was not phosphorylated (Fig. 5). However, the retention time of the Ser^{111} peptide from adipocytes could differ from that of the recombinant peptide if the COOH terminus of PHAS-I was modified in cells. To address this issue, adipocyte PHAS-I was phosphorylated by incubating heat-treated extracts of fat cells [18] with CK2 and [γ-^{32}P]ATP. Immunoprecipitation of the ^{32}P-labeled PHAS-I followed by digestion with lysyl endopeptidase yielded a single ^{32}P-labeled phosphopeptide that eluted from the reverse phase column in exactly the same position as the Ser^{111} peptide from recombinant PHAS-I (Fadden, Haystead, and Lawrence, unpublished observations). Thus, there does not seem to be a COOH terminal modification that alters the retention time of the Ser^{111} peptide. Moreover, even if such a modification did exist, ^{32}P should still be released in the seventh cycle when the Ser^{111} peptide was subjected to sequential Edman degradation. For this reason, our previous finding that none of the phosphopeptides derived from lysyl peptidase digestion of PHAS-I from ^{32}P-labeled adipocytes produced a surge in ^{32}P release in cycle 7 provides further support for the conclusion that Ser^{111} was not phosphorylated in adipocytes [7].

4. Discussion

Ser^{111} is the preferred site in PHAS-I for phosphorylation by CK2. This site is located near the COOH terminus of the protein, and it may be more accessible to the kinase than the other 3 sites that fit the minimal consensus for phosphorylation by CK2. Also, Ser^{111} is positioned just downstream of a pair of Glu residues [18]. Although an Asp/Glu residue in the +3 position is a strong determinant for phosphorylation by CK2, acidic residues on the NH_2 terminal side exert a positive influence [16,17]. Thus, the more acidic environment of Ser^{111} and its location near the COOH terminus of PHAS-I may explain why it is preferentially phosphorylated by the kinase.

Insulin has been reported to activate CK2 in rat adipocytes [14], and PHAS-I is clearly phosphorylated by the kinase in vitro. Moreover, CK2 has been shown to efficiently phosphorylate PHAS-I when the protein is bound to eIF4E [13,15]. In this respect CK2 differs from MAP kinase, which phosphorylates PHAS-I at a relatively slow rate when PHAS-I is bound to eIF4E [10,13]. Based on these findings it was logical to propose that phosphorylation of PHAS-I in the CK2 site primes PHAS-I in the complex with eIF4E for phosphorylation by other kinases [3]. However, the absence of phosphate in Ser^{111} in PHAS-I immunoprecipitated from rat adipocytes seems inconsistent with a role of this site in cells.

eIF4E is also controlled in 3T3L1 adipocytes by PHAS-II (also known as 4E-BP2) [6], a protein having approximately 60% amino acid sequence identity to PHAS-I [24]. PHAS-II contains neither a Ser nor Thr in the position corresponding to Ser^{111} in PHAS-I [6,24]. Consistent with the identification of Ser^{111} as the preferred site for CK2 in PHAS-I, PHAS-II is not phosphorylated by the kinase in vitro [15]. However, like PHAS-I, non-phosphorylated PHAS-II binds tightly to eIF4E [24], and when phosphorylated via the mTOR signaling pathway in response to insulin, PHAS-II dissociates from eIF4E [6]. The fact that PHAS-II is regulated even though it does not possess the equivalent of Ser^{111} provides additional support for the argument that Ser^{111} phosphorylation is not needed for the control of PHAS-I by insulin.

Acknowledgements: This work was supported in part by National Institutes of Health Grants DK52573 and DK28312.

References

[1] Lawrence Jr., J.C. and Abraham, R.T. (1997) Trends Biochem. Sci. 22, 345–349.
[2] Sonenberg, N. (1996) in: Translational Control (Hershey, J.W.B., Mathews, M.B. and Sonenberg, N., Eds.) pp. 245–249, Cold Spring Harbor Press, Cold Spring Harbor, NY.
[3] Proud, C.D. and Denton, R.M. (1998) Biochem. J. 328, 329–341.
[4] Lazaris-Karatzas, A., Montine, K.S. and Sonenberg, N. (1990) Nature 345, 544–547.
[5] De Benedetti, A. and Rhoads, R.E. (1990) Proc. Natl. Acad. Sci. USA 87, 8212–8216.
[6] Lin, T.-A. and Lawrence Jr., J.C. (1996) J. Biol. Chem. 271, 30199–30204.
[7] Fadden, P., Haystead, T.A.J. and Lawrence Jr., J.C. (1997) J. Biol. Chem. 272, 10240–10247.
[8] Beretta, L., Gingras, A.-C., Svitkin, Y.V., Hall, M.N. and Sonenberg, N. (1996) EMBO J. 15, 658–664.
[9] Graves, L.M., Bornfeldt, K.E., Argast, G.M., Krebs, E.G., Kong, X., Lin, T.-A. and Lawrence Jr., J.C. (1995) Proc. Natl. Acad. Sci. USA 92, 7222–7226.
[10] Lin, T.-A., Kong, X., Saltiel, A.R., Blackshear, P.J. and Lawrence Jr., J.C. (1995) J. Biol. Chem. 270, 18531–18538.
[11] Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence Jr., J.C. and Abraham, R.T. (1997) Science 277, 99–101.
[12] Brunn, G.J., Fadden, P., Haystead, T.A.J. and Lawrence Jr., J.C. (1997) J. Biol. Chem. 272, 32547–32550.

[13] Diggle, T.A., Moule, S.K., Avison, M.B., Flynn, A., Foulstone, E.J., Proud, C.G. and Denton, R.M. (1996) Biochem. J. 316, 447–453.
[14] Diggle, T.A., Schmitz-Peiffer, C., Borthwick, A.C., Welsh, G.I. and Denton, R.M. (1991) Biochem. J. 279, 545–551.
[15] Lawrence, J.C.J., Fadden, P., Haystead, T.A.J. and Lin, T.-A. (1997) in: Advances Enzyme Regulation (Weber, G., Ed.) pp. 239–267, Elsevier, New York, NY.
[16] Kennelly, P.J. and Krebs, E.G. (1991) J. Biol. Chem. 266, 15555–15558.
[17] Meggio, F., Marin, O. and Pinna, L.A. (1994) Cell. Mol. Biol. Res. 40, 401–409.
[18] Hu, C., Pang, S., Kong, X., Velleca, M. and Lawrence Jr., J.C. (1994) Proc. Natl. Acad. Sci. USA 91, 3730–3734.
[19] Haystead, T.A.J., Haystead, C.M.M., Hu, C., Lin, T.-A. and Lawrence Jr., J.C. (1994) J. Biol. Chem. 269, 23185–23191.
[20] Hathaway, G. and Traugh, J.A. (1983) Methods Enzymol. 99, 317–331.
[21] Roskoski, R. (1983) Methods Enzymol. 99, 3–6.
[22] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[23] Geer, P., Luo, K., Sefton, B.M. and Hunter, T. (1994) (Hardee, D.G., Ed.) pp. 31–59, University Press, Oxford.
[24] Pause, A., Belsham, G.J., Lin, T.-A., Lawrence Jr., J.C. and Sonenberg, N. (1994) Nature 371, 762–767.
[25] Russo, G.L., Vandenberg, M.T., Yu, I.J., Bae, Y.-S., Franza Jr., B.R. and Marshak, D.R. (1992) J. Biol. Chem. 267, 20317–20325.
[26] Lin, T.-A., Kong, X., Pause, A., Haystead, T.A.J., Belsham, G., Sonenberg, N. and Lawrence Jr., J.C. (1994) Science 266, 653–656.